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OBSERVATIONS ON THE STRUCTURE OF THE COCHLEAR DUCT LIMBUS OF REPTILES[1]

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Introduction

The wall of the membranous labyrinth of the vertebrate inner ear is composed of a remarkable material that is unique in the vertebrate body. The general nature and capacity of this material was well stated by de Burlet (1934), one of the outstanding contributors to the knowledge of the vertebrate inner ear. De Burlet states (our translation, p. 1326), "The labyrinth wall, besides the epithelium, consists of a connective tissue which gives the organ (the labyrinth) its characteristic firmness and elasticity. That the isolated labyrinth (when dissected free of the otic capsule) does not collapse but maintains its shape is attributable to this connective tissue. In structure and consistency it is reminiscent in many ways, of cartilage; Retzius (1881 and 1884) named it spindle cartilage because of the form of the cellular elements which it contains. This tissue is especially met with in the lower vertebrate classes, in fish as well as amphibia. The thickness and consistency of this cartilage-like wall layer varies from place to place in the same labyrinth. In general, the wall of the semicircular canals is thicker than that of the ampullae, utriculus, and sacculus. However, even in a single area there may be differences in the degree of development of this structure."

In the cochlear duct of reptiles, birds, and mammals, the papilla acustica basilaris (organ of Corti) is supported upon a basilar membrane which in turn

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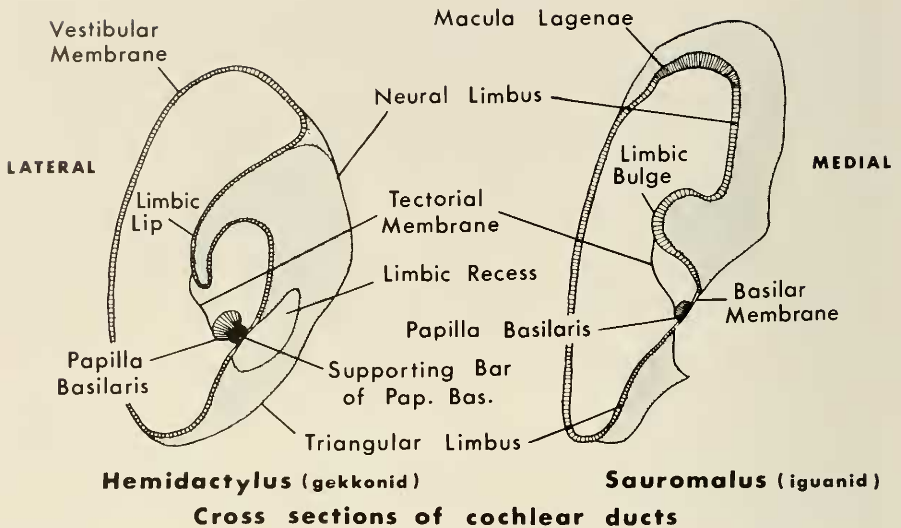

FIGURE 1.

is supported by a supporting framework known as the limbus (figs. 1, 2, and 3). In mammals, the limbus spiralis together with the spiral ligament is homologous with the avian and reptilian limbus. Kuhn as early as 1882, understood these relationships, and Shute and Bellairs (1953, p. 697) more recently point up these relationships between reptiles and mammals.

The basilar membrane (called papillary bar where it is thickened) is in reality continuous with and similar in nature to the limbic framework, differing only in the sparsity of cellular elements.

The cochlear limbus of the amniote vertebrates is made up of the same basic labyrinthine supporting tissue (referred to above) that is found in all vertebrates, but in this location shows certain modifications. Iurato (1962), in a report of the ultrastructure of this supporting tissue in rat cochlea, proposes that this supporting tissue is epidermal in origin rather than mesodermal as is the case with connective tissue.

Because the limbus is the basic supporting element of the Corti organ, intimate knowledge of its structure and evolution are important in an analysis of the acoustic mechanism.

Apparently at no time in the earlier studies of the mammalian cochlea was the limbus ever described as being cartilaginous or even cartilage-like in nature. In reptiles and birds, however, the cochlear limbus was described as a type of cartilage. Otto Deiters (1862) refers to the limbus as a cartilaginous frame, but his drawings of the limbus of *Lacerta agilis* reveal its non-cartilaginous nature.

Kuhn, in 1882, while referring to the cartilage mass and cartilage limbs of the cochlear duct wall as "spindle-cartilage," describes it as a homogeneous ground substance in which are found partly round, and partly spindle-shaped cells.

Even as recently as 1926 the noted student of labyrinth structure, H. Held, refers to the avian limbus as the "Knorpelrahmen" even though his meticulous drawings reveal its non-cartilaginous nature.

As noted above, de Burlet (1934) was of the opinion that the limbus was modified connective tissue, and in 1953 Shute and Bellairs pointed out the undesirability of using such terms as "Knorpelschenkel" and cochlear cartilage since the limbus is not typical cartilage.

Hamilton (1964) refers to the limbic structure of lizards as a dense, highly organized periotic connective tissue.

In order to determine whether the reptilian limbus is or is not structurally similar to cartilage and whether it is similar or not to mammalian limbus, certain histological, histochemical, and ultrastructural studies were carried out on a variety of reptilian species. Because the limbus is the basic supporting element of the Corti organ, intimate knowledge of its structure and evolution are important in understanding the hearing mechanism.

Methods and Materials

The gross anatomical features of cochlear duct limbi of a large variety of reptiles has been studied and reported upon (Miller, 1966a, 1966b). As reported earlier (Miller, 1966a) the intact cochlear duct of a reptile may be easily dissected out of the otic capsule. In most cases the cochlear duct was preserved in 10 per cent formalin or 70 per cent ethyl alcohol for gross study.

For histological studies of cochlear duct structure, experience showed that *in situ* fixation followed by subsequent dissection was better than removing the cochlear duct before fixation. The lateral wall of the otic capsule was removed by rapid dissection exposing the lateral face of the cochlear duct. The entire head was then placed in fixative and after fixation the cochlear duct was dissected free of the cochlear recess. In the case of very small animals, the heads were merely cut off and dropped into the fixative.

For histological studies the tissues were fixed in 10 per cent neutral formalin, Bouin's alcohol-formalin, and acrolein. The cochlear ducts were embedded in either paraffin or celloidin. Superior preservation of cytological detail was achieved with celloidin embedding. Sections were cut at 3 to 6 μ.

For histochemical studies whole heads of *Microgecko helenae* and *Ablepharus gravanus* were fixed in 10 per cent neutral formalin and decalcified in Jenkin's fluid (4 per cent Hydrochloric acid and 3 per cent Acetic acid in 95 per cent

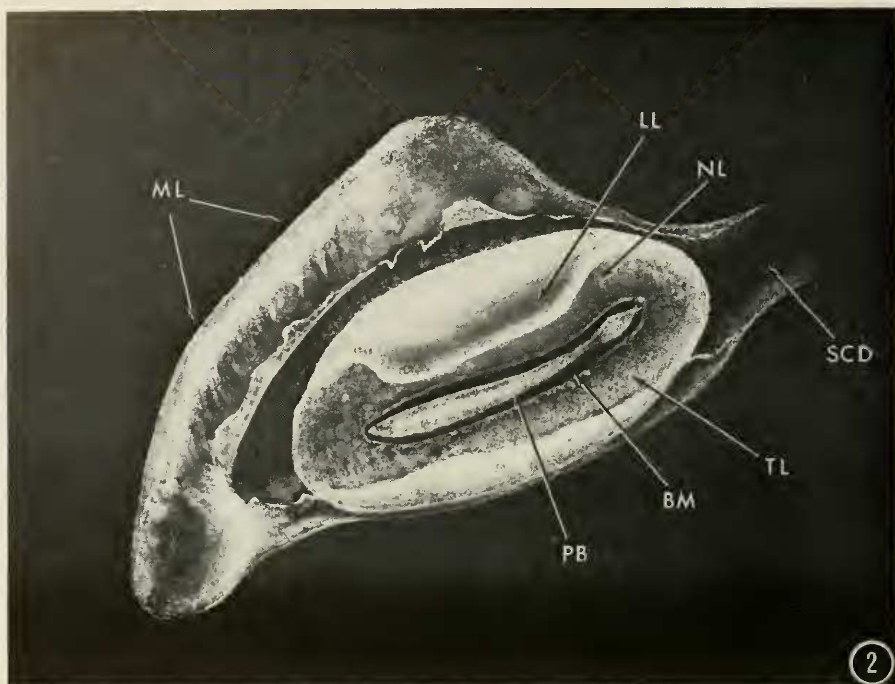

FIGURE 2. Drawing of the lateral view of the cochlear duct of *Cnemidophorus tigris*. The basilar membrane (BM) is stretched across the central opening in the limbus and supports the papilla basilaris (PB). NL, neural limbus; TL, triangular limbus; LL, limbic lip; ML, macula lagenae; SCD, sacculo-cochlear duct.

alcohol, chloroform, and water). They were then post-fixed in 10 per cent formalin, embedded in paraffin, and sectioned at 6 to 7 μ.

A variety of staining procedures were used:

For general structure:

1. Hematoxylin and Eosin
2. Iron-Hematoxylin-Aniline-Blue (Koneff, '36)

For special structures:

3. Gordon and Sweet Silver Method for Reticular Fibers
4. Acid Orcein for Elastic Fibers
5. Verhoeff's Method for Elastic Fibers

For histochemical studies:

6. PAS Stain (after McManus)
7. Alcian Blue Stain (after Steedman)
8. Toluidine Blue for Metachromasia (Kramer and Windrum)
9. Colloidal Iron Test (Burn modification of Mowry's Method)
10. Methylene Blue Extinction Test

For ultrastructural studies several living specimens of *Gerrhonotus multicarinatus* and *Lygosoma lateralis* were prechilled to 4°C. The dissection was the same as described above except that the cochlear duct was not fixed *in situ*. Rather, the cochlear duct was removed immediately and immerged in cold fixative. This operation took 4 minutes. The tissue was fixed for 1 hour in either Dalton's 3 per cent glutaraldehyde or paraformaldehyde-glutaraldehyde, washed 1 hour in 0.2 M sodium cacodylate buffer, pH 7.5, and post-fixed for 2 hours in Palade's 1 per cent osmium tetroxide. It was flat-embedded in Araldite and sectioned with glass knives on an LKB ultratome. The sections were mounted on unsupported grids, stained with saturated uranyl acetate from 10 to 30 minutes, and also stained with lead citrate from 2 to 15 minutes. Sections were viewed in an RCA 3F electron microscope with magnifications ranging from × 2000 to × 30,000.

OBSERVATIONS

The gross anatomy of the cochlear duct of reptiles has been described in detail in Miller 1966a and 1966b. As shown in these papers, the reptilian cochlear duct is a roughly pyramid-shaped sac connected to the posteroinferior aspect of the sacculus by the sacculo-cochlear duct. The anterodorsal, medial, and posteroventral walls of the cochlear duct are made up of a modified labyrinthine supporting tissue, while the lateral wall (Reissner's membrane) is epithelial (figs. 1, 2, and 3).

A portion of the medial wall of the duct is thickened and forms a ring-like structure surrounding the basement membrane on which rests the papilla basilaris (fig. 2). This specialized area of labyrinthine supporting tissue surrounding the basement membrane is the so-called limbus and is homologous with the combined limbus spiralis and spiral ligament of the mammalian cochlear duct. The reptilian limbus varies in shape from that of a thin saucer to a heavy variously sculptured ovoid or elongate rim.

In lizards the cochlear duct is never conjoined to the sacculus, but in snakes and turtles the upper (anterodorsal) wall of the cochlear duct is often fused with the ventrolateral wall of the sacculus.

The structure of the limbus of the reptilian cochlear duct is basically similar to the supporting tissue of the labyrinth in general, but in this location it is thicker (more dense). The following description applies essentially to the modified labyrinthine supporting tissue in the limbic area only.

Since the primary object of this communication is to compare the limbic tissue with cartilage, the structure and histochemistry of these two tissues are reported side by side below:

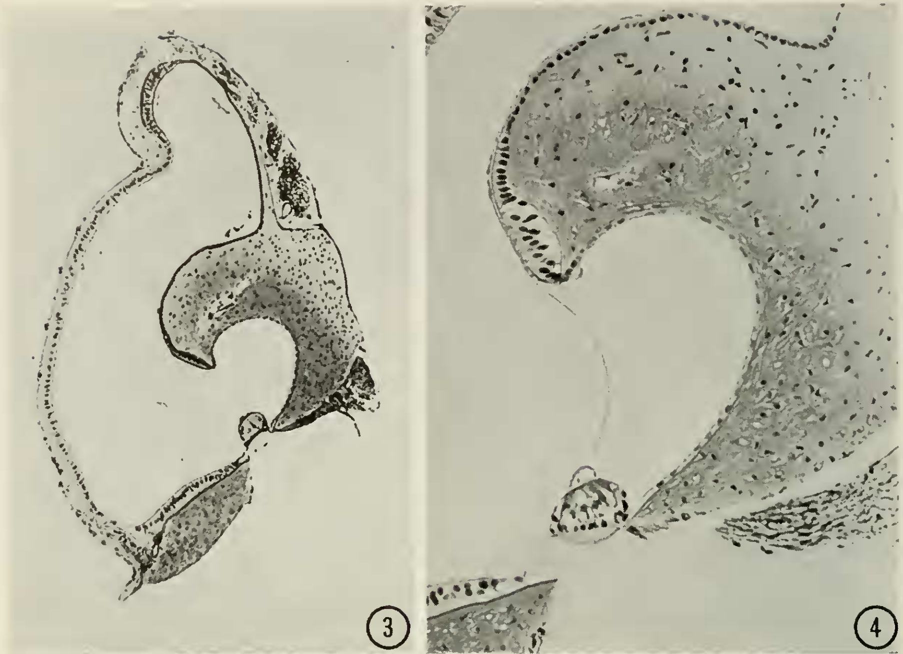

FIGURE 3. Cross-section of cochlea of an *Ameiva* species. IHAB Stain. × 75. Compare with figure 1.

FIGURE 4. Cross-section of cochlea of an *Ameiva* species taken at higher power to show variation in shape of cells in the limbic supporting structure. The clear streaks seen between the cells are fragments of cytoplasmic processes. IHAB Stain. × 200.

COMPARISON BETWEEN CARTILAGE AND DENSE PERIOTIC SUPPORTING TISSUE

Cartilage	Dense Periotic Supporting Tissue
1. Cells	
Usually somewhat spherical in shape and tending to form groups or nests of two to four cells	Exhibit great variation in shape from ovoid to stellate with long processes extending irregularly into matrix
2. Intercellular substance	
Fibers are of two types: large mature collagen fibrils with 640A. periodicity intermixed with microfibrils which are about 100A. in diameter and without periodicity	Only microfibrils 125A. in diameter without periodicity
3. Peripheral related cells	
Almost always covered with connective tissue (perichondrium)	Related to epithelial covering on one side and supporting tissue elements on the other
4. Vascular Channels	
Occur but rarely	Vascular channels more frequently present

5. Elastic fibers	
Absent in hyaline, but present in elastic cartilage	None
6. Reticular fibers	
None	None
7. Aldehyde Fuchsin Stain	
Stained very deeply purple	Not stained
8. Basophilia	
While basophilic, the basophilia may be greater about the capsules due to greater concentration of chondromucoid	No increase in basophilia about cellular elements
9. PAS Reaction	
Positive	Positive but less intense than cartilage
10. Metachromasia-Gamma	
Positive	Negative
11. Methylene Blue Extinction	
Below pH 4	Above pH 4
12. Alcian Blue Stain	
Stained around capsules	No staining
13. Colloidal Iron Test	
Strong positive	Weak positive

Since whole head sections were used for histochemical studies we were able to compare the reactions in cartilage and in limbic supporting tissue on the same slides. Both structurally and chemically limbic tissue differs from cartilage.

The cellular elements of limbic tissue, unlike those of cartilage, remain single or ungrouped and vary greatly in shape. The cells may be ovoid or spindle-shaped or even stellate with long irregular processes extending into the matrix (figs. 4, 5, and 6). The matrix appears to be irregularly traversed by many fine cytoplasmic processes. Cartilage cells, on the other hand, tend to be spheroidal and often form nests or groups of two to four cells.

Chemically, chondroitin sulfates and hyaluronic acid are the major acid mucopolysaccharide (AMPS) components of cartilage. AMPS in cartilage are readily demonstrable by their gamma-metachromasia with toluidine blue, their affinity for Alcian Blue Stain and by their ability to absorb colloidal iron. The content of AMPS in limbic tissue was lower and demonstrable only by the Colloidal Iron Test.

In the Colloidal Iron Test, labile factors of the AMPS was demonstrated with Wydase Testicular Hyaluronidase.[2] Both sections treated and untreated with hyaluronidase prior to colloidal iron absorption showed positive reactions, the treated sections showing much less reaction than the untreated sections.

The ultrastructural observations of the limbus revealed individual cells separated from each other by an intercellular matrix in which slender fibrils are oriented at random (figs. 7, and 8). At the junction of the limbus with the

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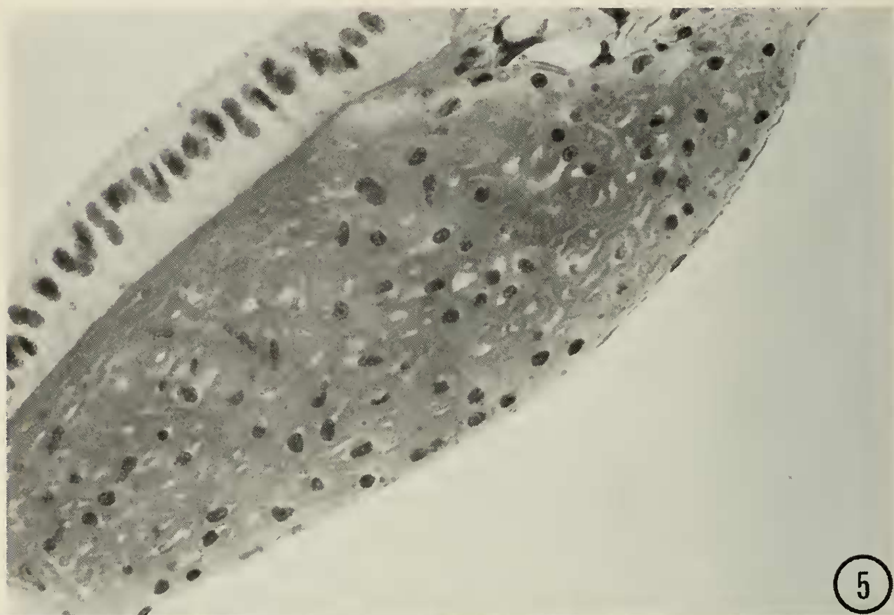
[2] Wyeth Laboratory, Inc.



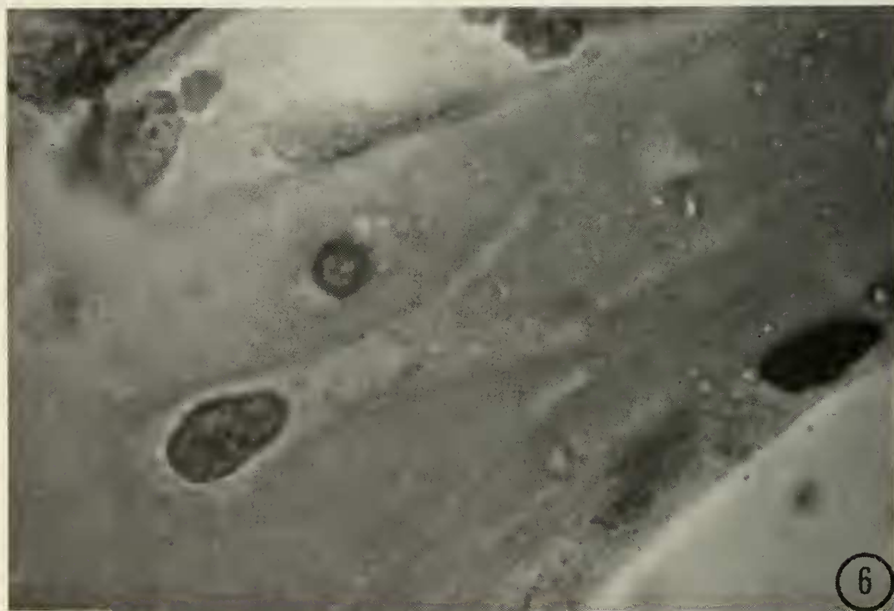


FIGURE 5. High power view of triangular limbus of an *Ameiva* species showing irregularity of cells. IHAB Stain. × 490.

FIGURE 6. Higher magnification of cells in the neural limbus of an *Ameiva* species, one of which shows long cytoplasmic processes. IHAB Stain. × 2700.

basilar membrane the fibers show some orientation as they converge from the relatively thick limbus into the thin basilar membrane. In the basilar membrane and its thickened portion, the papillary bar, the microfibrils show a tendency to associate in bundles which are predominantly oriented in a plane at 90 degrees to the long axis of the basilar membrane. The microfibrils are approximately 125A. wide when measured in longitudinal section (figs. 9, and 10). They are comparable in size to neurofilaments (fig. 8). When cut in cross-section they have a circular profile and are slightly wider. These fibers do not exhibit cross-banding nor the 640A. periodicity of typical collagen. However, in some micrographs the fibers appear beaded. Neither wide fibers with typical collagen banding and 640A. periodicity nor elastic fibers were seen in the limbus.

The cellular population showed variations which probably indicate different functional states of the cells. The active cells have a moderate amount of cytoplasm with a well developed endoplasmic reticulum and Golgi complex (fig. 7). The cytoplasm and cell organelles are greatly reduced in the presumably less active cells.

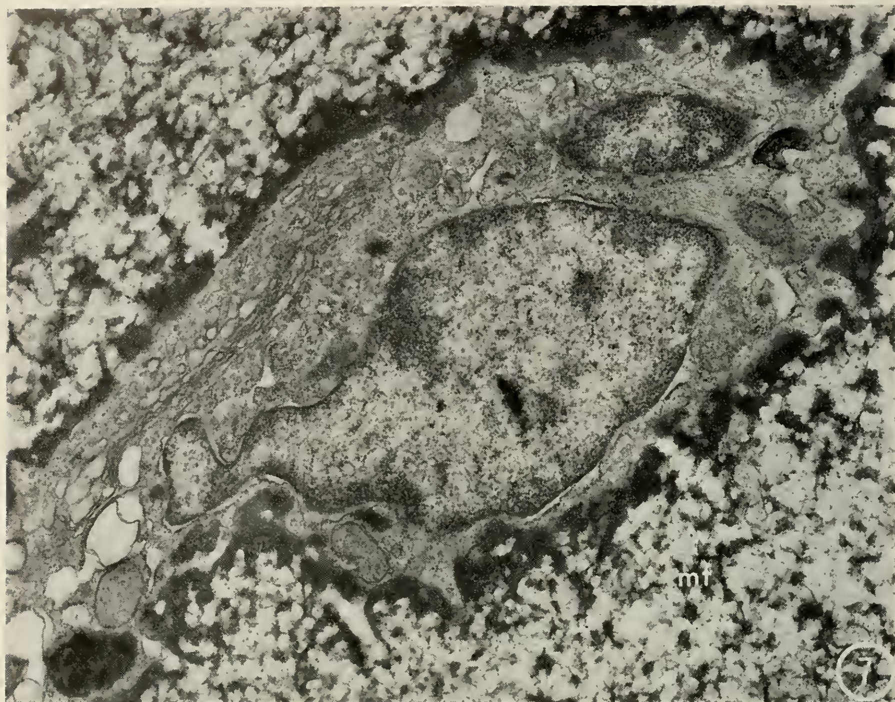
Discussion

Histology. Cartilage cells show some variation in shape according to their location. Those near the perichondrium or free joint surfaces become somewhat flattened parallel to the surface, and those in deeper layers are flattened on their sides which are contiguous with other cells because of mutual pressure. However, they are usually spheroidal and occur frequently in spherical nests of two to four cells with well defined contours. The limbic cells, on the other hand, show great irregularity in shape with long slender branching processes which extend far into the matrix.

Histochemistry. The positive PAS reaction in paraffin sections after removal of glycogen is indicative of the presence of carbohydrate-protein complexes, which include neutral mucopolysaccharide and muco- and glycoproteins. It has been demonstrated that acid mucopolysaccharides do not react positively to PAS stain (Pearse, 1960). The positive PAS in cartilage is due to chondromucoid, a glycoprotein, present in the ground substance.

The ground substance in reptilian limbic tissue consists mainly of muco- or glycoproteins as demonstrated by the diastase-fast PAS reaction. Plotz and Perlman (1955) describe the presence of a very insoluble glycoprotein in the ground substance of the basilar membrane, spiral limbus and spiral ligament of bat cochlea. It was demonstrated as a Hotchkiss (PAS) positive material in freeze-dried tissue.

The acid mucopolysaccharides (AMPS) in limbic tissue was demonstrated by the Colloidal Iron Test. Both hyaluronidase fast (hyaluronic acid, chondroitin sulfates A and C) and hyaluronidase labile (chondroitin sulfate B) factors were present although to a far less degree than in cartilage.



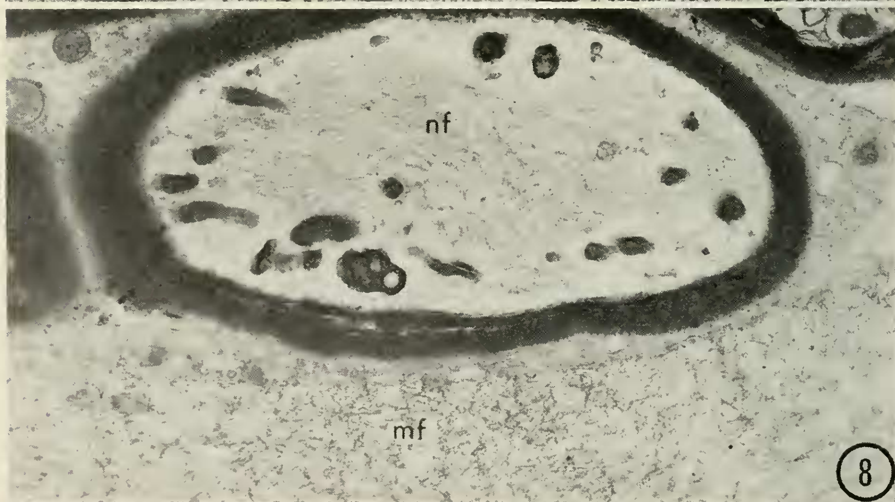


FIGURE 7. A cell in the neural limbus of *Gerrhonotus multicarinatus.* Microfibrils (mf) are present in the surrounding matrix. Glutaraldehyde. × 16,400.

FIGURE 8. A myelinated nerve beneath the neural limbus of *Lygosoma lateralis.* Note the comparable size of the neurofilaments (nf) and the microfibrils (mf). Paraformaldehyde-glutaraldehyde. × 6760.

Tests for metachromasia and methylene blue extinction as well as the Colloidal Iron Test and Alcian Blue Staining Method are significant in differentiating the muco- or glycoproteins from the AMPS. It is possible that the amount of AMPS in the limbic structure of reptiles is not of sufficient quantity to be demonstrable by these methods except by the Colloidal Iron Test. The AMPS in cartilage were clearly demonstrated by all of these methods.

The histochemistry of the guinea pig cochlea was studied by Mangabeira-Albernaz (1961). By employing the Alcian Blue Stain and Colloidal Iron Test he showed more AMPS in the guinea pig limbus than are found, relatively, in lizard limbus.

ULTRASTRUCTURE. Karrer (1958) reported slender filaments approximately 110A. in diameter in the loose connective tissue of the tunica propria in mouse bronchiole. Some of the filaments were beaded but due to lack of contrast and resolution the dimensions of the cross-structure could not be determined.

Bairati *et al.*, (1964) indicated the presence of very slender fibrils, 80A. in diameter without periodic structure, intermixed with typical collagen fibers in the reticulum of the mammalian lymph node. Thin fibers were noted more frequently in young animals.

Typical collagen fibers have approximately a 640A. periodicity with characteristic cross-banding.

Zelander (1959) noted the presence of fine (85A. wide) unbanded fibrils in the matrix of adult guinea pig and mouse articular cartilage. These fine fibrils were located near the chondroblast. As one moved farther away from the cell, the fibers became thicker and showed 640A. periodicity.

Scott and Pease (1956) described fibers of 100A. diameter and lacking periodicity in the matrix of epiphyseal cartilage in kittens.

Sheldon (1964) summarizes the evidence for fine fibrils in a variety of cartilage and concludes that the periodic banded structure of the collagen fibril is not a typical feature of cartilaginous collagen.

Iurato (1962) reported the presence of extremely slender filaments having a diameter of about 100A. and of microglobular appearance in the supporting structure of the organ of Corti in the rat. These filaments are the elementary component in the various filamentous laminae, bundles or fibers in the supporting structure. Chemical, diffractographic, and morphological tests indicated that these filaments were composed of protein materials which were not collagen or elastin and could perhaps broadly be classified in the keratin, epidermin, myosin, fibrinogen group of proteins.

The intercellular fibrils in the limbus of *Gerrhonotus* and *Lygosoma* can be characterized as slender unbanded microfibrils which occasionally show a beaded appearance. These microfibrils seem to be structurally similar to the slender fibrils previously described in various types of connective tissue and in the supporting structure of the organ of Corti in the rat. Whether these filaments

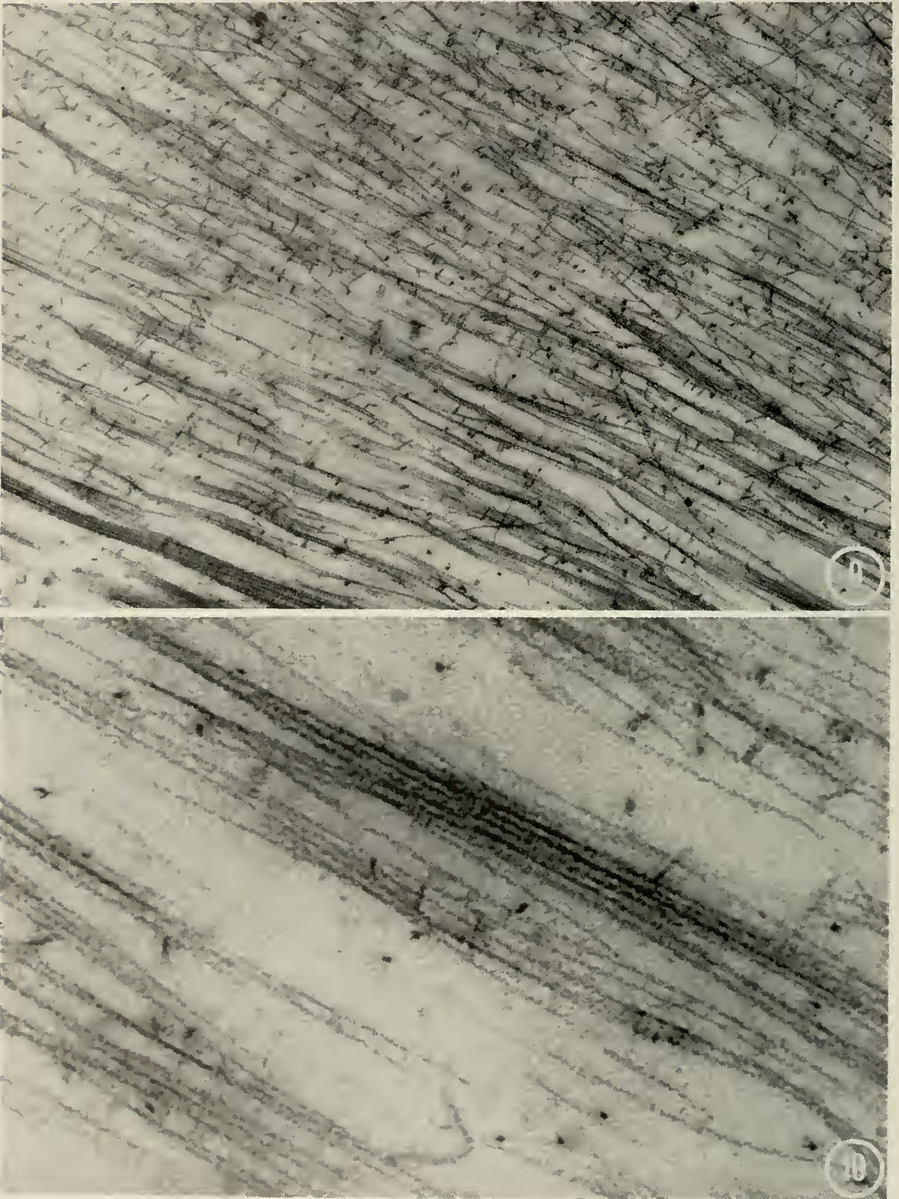


FIGURE 9. Microfibrils in the papillary bar of the basilar membrane of *Lygosoma lateralis*. Paraformaldehyde-glutaraldehyde. × 21,000.

FIGURE 10. Same as figure 9 at higher magnification. Microfibrils have an average diameter in longitudinal section of 125A. They do not show periodicity except for a slightly beaded appearance. Paraformaldehyde-glutaraldehyde. × 88,500.

are collagen which have not polymerized to a diameter sufficient to show typical 640A. periodicity, or whether they are a keratin-like protein as Iurato suggests for filaments in the mammalian limbus, has not yet been determined.

The fact that none of the microfibrils polymerized to a diameter sufficient to show typical collagen periodicity and the homology between the supporting structure of the organ of Corti of reptiles and mammals favor interpreting these fibrils not as collagen or elastin but as a protein falling broadly into the keratin, epidermin, myosin, fibrinogen group. Therefore, the tissue cannot be classified as cartilage or as standard fibrous connective tissue.

Summary

The reptilian cochlear duct limbus which supports the basilar membrane on which rests the organ of Corti is homologous with the spiral ligament together with the spiral limbus of the mammalian cochlear duct. The limbic structure in reptiles and birds has been variously and erroneously reported in the past as cartilaginous or cartilage-like in nature.

Recently, Iurato has proposed that this supporting tissue is epidermal in origin rather than mesodermal as is the case with connective tissue.

The nature of this limbic supporting tissue is described and compared with that of cartilage by means of histological, histochemical, and ultrastructural studies.

Histologically, the cells of the limbic structure exhibit a great variation in shape from oval to stellate with irregular elongate processes. The cells always occur singly. Cartilage cells are more or less spherical and often form nests or groups of two to four cells.

Histochemically, the limbic tissue showed a far smaller amount of AMPS than was demonstrated in cartilage. The ground substance appears to consist mainly of muco- or glycoproteins.

Ultrastructurally, the intercellular substance of the limbus contains microfibrils of approximately 125A. diameter without periodicity. Typical mature collagen fibers with 640A. periodicity are absent. The limbic microfibrils are structurally similar to the non-collagenous microfibrils in the supporting tissue of the mammalian organ of Corti.

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